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(57) Abstract

Polypeptides with specific binding affinity for glucan - especially the glucan-binding domain of glycosyl transferase enzyme - is utilised in a composition for oral care. The polypeptide may block the binding sites in dental plaque where glycosyl transferase would bind and generate more plaque, or it may be conjugated to - and provide targeted delivery of - an antiplaque or antistain agent.

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GLUCAN-BINDING PROTEINS, AND USE THEREOF

Field of the Invention

The present invention relates to polypeptides with glucan-binding properties, to hybrid materials containing such polypeptides, and to novel active systems targeted to dental plaque and oral care compositions containing same.

Background of the Invention

The initiation and accumulation of dental plaque results from the adherent attachment of bacteria and their products to the teeth. Attachment of many plaque bacteria is mediated through the production of adhesive extracellular polysaccharides from dietary sucrose. The main two such polysaccharides synthesised from sucrose in dental plaque are glucans and fructans *[Hamada S and Slade H D 1980 Biology, immunology and cariogenicity of Streptococcus mutans Microbiol. Rev. 44: 331-384]*. These adhesive polysaccharides are known to increase attachment of plaque bacteria by providing stereochemically specific binding sites, and also through non-stereospecific trapping of bacteria. The bacterial enzymes which synthesise glucans and fructans, referred to as glucosyltransferases (GTFs) and fructosyltransferases (FTFs) respectively, are secreted by many plaque bacteria. They are found associated with bacterial surfaces, adsorbed to tooth surfaces, and in the saliva which bathes the oral cavity *[Rolla G, Ciardi J E, Eggen K, Bowen W H and Afseth J (1983) Free glycosyl- and fructosyltransferase in human saliva and adsorption of these enzymes to teeth in vivo. In: R J Doyle and J E Ciarid (ed) Glucosyltransferase, glucans, sucrose and dental caries. Spec. Suppl. Chem. senses. IRL Press Washington DC p 21-30]*.

Glucans in plaque are composed predominantly of alpha 1,6-linked and alpha 1,3-linked glucose residues.

Polysaccharide-mediated bacterial attachment is considered to be crucial to the

accumulation of pathogenic dental plaque *[Hamada and Slade, 1980].* Several species of oral bacteria produce proteins which recognise and bind to glucans. These proteins include the glucosyltransferases (GTFs) which are involved in forming plaque and also non-enzymatic glucan-binding proteins (GBPs). GTFs and GBPs specifically recognise glucans containing alpha 1,6-linked glucose residues, and mediate bacterial binding to extracellular plaque matrix *[Schilling K M and Bowen W H 1992 Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for* <u>*Streptococcus mutans*</u> <u>*Infect. Immun.*</u> *60; 284-295].*

Molecular analysis of a GBP and several GTFs from oral streptococci has shown that these proteins have distinct domains (GBDs) which are responsible for stereospecific binding to glucans *[Wong C, Hefta S A, Paxton R J, Shively J E and Mooser G (1990) Size and subdomain Architecture of the glucan binding domain of sucrose; 3 α-D glucosyltransferase from* <u>*Streptococcus sobrinus*</u>*. Infect. Immum. 58; 2165-2170; Ferretti J J, Gilpin M L and Russell R B (1987) Nucleotide sequence of a glucosyltransferase gene from* <u>*Streptococcus sobrinus*</u> *MEF28,* <u>*Journal Bact.*</u> *169, 4271-4278; Kato C and Kuramitsu H K (1990) Carboxyl-terminal deletion analysis of the* <u>*Streptococcus mutans*</u> *glucosyltransferase-I enzyme. FEMS Microbiol. Lett 72, 299-302].* Studies with glucan-binding domains have shown that these bind to glucans with affinities similar to that observed for intact GTFs.

Other polysaccharide-binding domains are known and have been conjugated to other proteins and peptides to aid in downstream processing of recombinant fermentation products. For instance, researchers have made fusion proteins containing starch-binding domains *[Chen L, Ford C and Nikolov Z (1991) Adsorption to starch of a ß-galatosidase fusion protein containing the starch-binding region of Aspergillus glucoamylase* <u>*Gene*</u> *99l, 121-126]* and cellulose-binding domains *[Ong E, Greenwood J M, Gilkes N R, Kilburn D G, Miller R C and Warren R A J (1989) The cellulose-binding domains of cellulases; tools for biotechnology* <u>*TIBTech*</u> *7; 239-243]* for the purpose of purifying the proteins on starch and cellulose resins.

One of the major technical problems associated with the development of effective active systems for oral care benefits is obtaining substantive delivery of the agents to the desired site in the mouth (eg: plaque). For the most part, substantive anti-plaque agents currently in use are positively charged microbicides, such as bis-biguanides and quaternary ammonium compounds, which bind to oral surfaces through electrostatic interactions.

However, binding of cationic microbicides is non-specific in nature (as it is for hydrophobic agents as well); these agents bind to all oral tissues. Furthermore, use of cationic molecules such as chlorhexidine and cetyl pyridinium chloride in dentifrices and oral rinses has been associated with tooth staining and undesirable product taste.

Summary of the Present Invention

The present invention uses glucan-binding polypeptides to target and bind to glucans either in plaque matrix or associated with bacterial surfaces. The glucan-binding polypeptides can be conjugated to an anti-plaque or other agent and used to deliver this to plaque, providing substantivity and often reducing the amount of agent needed. They can avoid the negative aspects associated with cationics and other non-specific oral care active agents. Conjugates can be formed with a variety of materials, either chemically or through the tools of molecular biology (i.e. fusion proteins).

Broadly, in a first aspect, the present invention provides a polypeptide with specific binding affinity for glucan, which is not incorporated within a glycosyltransferase enzyme and desirably is covalently chemically bound to a further material which does not display glycosyltransferase enzymic activity.

The present invention's use of a glucan-binding polypeptide conjugated to another moiety is distinctive in several ways. First, conjugates described herein are derived from recognition systems involving alpha 1,6-linked glucans. Second, the conjugates described herein are used to provide a benefit in the oral environment. Third, the glucan-binding

conjugates are intended to target to a biofilm where they deliver secondary physiological effects in a biochemically hostile environment, as opposed to the described use of other polysaccharide-binding domain fusions to bind to simple chromatographic resins *in vitro*.

It is also a possibility within this invention to use glucan-binding polypeptides to block binding sites on existing glucan. Polymer synthesis by GTF requires glucan-binding by the enzyme. Glucan-binding polypeptides will compete with GTFs for binding sites and thus act as stereospecific inhibitors of GTF-catalysed glucan synthesis in plaque. This will decrease the build-up and tenacity of plaque. An analogous possibility is that glucan-binding polypeptides will block sites in plaque matrix to which oral bacteria will bind, and in this way inhibit the build-up of plaque. If a glucan-binding polypeptide is used as a competitive inhibitor in this way, rather than as a means of targeting some other agent, it is nevertheless likely that the glucan-binding polypeptide will be fused to some other peptide residue which was needed for the synthesis step.

In a second aspect this invention provides a composition for topical application in the mouth, comprising a polypeptide with specific binding affinity for glucan, in a carrier vehicle which is acceptable for use in the mouth.

Detailed Description of the Invention

It is a purpose of the present invention to utilise GBDs or other glucan-binding polypeptides or other glucan-binding polypeptides as targeting groups for the substantive delivery of oral care active agents, especially anti-plaque and anti-stain agents to the plaque on human teeth. Conjugates can be formed through chemical conjugation between glucan-binding polypeptide and a large number of agents by ester, sulfhydryl, peptide, isopeptide, amide and other types of chemical bonds. Entities which may be conjugated in one way or another, include organic compounds, inorganic complexes, proteins, enzymes, peptides, antibodies and various ligands. The conjugates can also be fusion or hybrid proteins produced by recombinant DNA technology.

Enzymes to be conjugated to glucan-binding polypeptides include but are not limited to oxidases, peroxidases, proteases, glycosidases, lipases, esterases, amidases, deaminases, ureases and polysaccharide hydrolases. In particular, oxidases can function as cytotoxic agents acting against the microbial species in dental plaque. Glucose and galactose oxidases produce hydrogen peroxide which is cytotoxic. Peroxidases can correct this to hypohalite, which is even more toxic. Both hydrogen peroxide and hypohalite are short-lived in vivo, and are made more effective by creation at their intended point of action, as provided by this invention.

Such enzymes can also function as anti-stain agents, since their products are bleaching species. Non-enzymatic anti-microbial proteins and peptides such as antibodies, antibody fragments, histatins, lactoferrin, defensins, magainins, cecropins, other cationic antibacteriocins and bacteriocins can also be conjugated to glucan-binding polypeptides. Microbicides including but not limited to triclosan, chlorhexidine, quaternary ammonium compounds, chloroxylenol, chloroxyethanol, thymol and fluoride can also be chemically conjugated to glucan-binding polypeptide. Anti-microbial cations such as Zn, Sn, Cu and others can be complexed to glucan-binding polypeptide by forming conjugates with appropriate chelating agents such as (poly) carboxylic acids, amino acids and so on. Targeting systems can also be produced by biotin-avidin conjugates. For instance biotin can be chemically conjugated to GBD which targets it to plaque where the biotin acts as a specific binding site for avidin-conjugates. Similarly, avidin-GBD conjugates can be used as plaque-specific binding sites for biotin conjugates.

It is also possible within this invention to use glucan-binding polypeptides as targeted release agents by conjugating anti-plaque or other oral care active agents to glucan-binding polypeptides with bonds which are sensitive to hydrolase, pH or oxidation/reduction. Fusions of a protein to glucan-binding polypeptide can be linked by protease-sensitive linker peptides which will be hydrolysed by endogenous or exogenous protases in plaque. Non-proteinaceous microbicides can be conjugated by esterase, amidase, lipase or other

hydrolase-sensitive bonds. Release can also come by the use of pH sensitive bonds or by conjugating proteins, peptides or other agents through S-S bonds which are sensitive to chemical reduction as the redox potential drops in plaque or as reducing agents accumulate.

Oral care products containing glucan-binding polypeptides can be in a variety of forms including toothpastes, gels, mouthwashes, powders, gargles, solutions, lozenges, chewing gum and dental floss.

The oral composition may furthermore comprise conventional ingredients, such as pharmaceutically acceptable carriers like starch, sucrose, polyols, surfactants, water or water/alcohol systems etc. When formulated into a dentifrice, such formulation may contain usual dentifrice ingredients. Thus, they may comprise particulate abrasive materials such as silicas, aluminas, calcium carbonates, dicalciumphosphates, hydroxyapatites, calcium pyrophosphates, trimetaphosphates, insoluble hexametaphosphates and so on, usually in amounts between 5 and 60% by weight.

Furthermore, the dentifrice formulations may comprise humectants such as glycerol, sorbitol, propyleneglycol, lactitol and so on.

Surface-active agents may also be included such as anionic, nonionic, amphoteric and zwitterionic synthetic detergents. Examples thereof are sodiumlaurylsulphate, sodium dodecylbenzenesulphonate, sodium mono- and dioctyl-phosphate, sodiumlauroylsarcosinate, cocamidopropylbetain

Binders and thickeners such as sodium carboxymethyl-cellulose, xanthan gum, gum arabic etc. may also be included, as well as synthetic polymers such as polyacrylates and carboxyvinyl polymers such as Carbopol®.

Flavours such as peppermint and spearmint oils may also be included, as well as preservatives, opacifying agents, colouring agents, pH-adjusting agents, sweetening agents

and so on.

Additional anti-bacterial agents may also be included such as Triclosan, chlorhexidine, copper-, zinc- and stannous salts, such as copper sulphate, zinc citrate and stannous pyrophosphate, sanguinarine extract, metronidazole. Further examples of additional anti-bacterial agents are quaternary ammonium compounds such as cetylpyridinium chloride; bis-guanides such as chlorhexidine digluconate, hexetidine, octenidine, alexidine; halogenated bisphenolic compounds such as 2,2' methylenebis-(4-chloro-6-bromophenol).

Polymeric compounds which can enhance the delivery of active ingredients such as the anti-bacterial agents can also be included. Examples of such polymers are copolymers of polyvinylmethylether with maleic anhydride and other similar delivery enhancing polymers, e.g. those described in DE-A-3,942,643 (Colgate)

Furthermore anti-inflammatory agents such as ibuprofen, flurbiprofen, aspirin, indomethacin etc. may also be included.

Anti-caries agents such as sodium- and stannous fluoride, aminefluorides, sodium monofluorophosphate, calcium lactate and/or calcium glycerophosphates, strontium salts and strontium polyacrylates, casein and casein digests and phosphoproteins may also be included.

Other optional ingredients include vitamins such as Vitamin C, plant extracts, potassium salts such as potassium citrate, potassium chloride and potassium nitrate.

Other optional ingredients include enzymes such as dextranase and/or mutanase, amyloglucosidase, glucose-oxidase with lactoperoxidase, neuraminidases, and hydrogen peroxide generating compounds such as potassiumperoxydiphosphate.

Furthermore, the oral compositions may comprise anti-calculus agents such as alkalimetal

pyrophosphates, hypophosphite-containing polymers, organic phosphonates, phosphocitrates etc.

Other optional ingredients that may be included are e.g. bacteriocins, bacteriophages, tissue respiratory factors, antibodies, bleaching agents such as peroxy compounds, effervescing systems such as sodium bicarbonate/citric acid systems, colour change systems, and so on.

Brief description of the Drawings

Fig. 1 diagrammatically illustrates the structures of the plasmid pUC18 38Hind and derivatives constructed from it. Restriction sites (where relevant) are shown in this and some subsequent figures with abbreviated identities as follows: B=*Bam*HI; Bgl=*Bgl*II; E=*Eco*RI; H=*Hind*III; K=*Kpn*I; P=*Pst*I; Pvu=*Pvu*II, S=*Sca*I, and X=*Xba*I. Restriction sites without abbreviation denote a *Bst*YI boundary site of two DNA fragments created following *Bam*HI/*Bgl*II ligation.

Fig. 2 illustrates the general strategy of the "heterodimer" system used to make intermediates for use in transformation.

Fig. 3 illustrates structure of the plasmids used for introduction of the integration anchor sites into the *S.gordonii* chromosome. Restriction sites are shown with abbreviated identities as described under Fig. 1; also Bs=*Bst*BI; C=*Cla*I; and Xh=*Xho*I.

Fig. 4 is a series of restriction maps which illustrate integration of the hybrid *GBD* gene into the *S.gordonii* chromosome. The topmost map shows chromosomal structure around the *gtfG* gene in *S.gordonii*. The subsequent maps, which are on a larger scale, show chromosomal structures of wild type (A), and primary (C), secondary (E), and GBD$^+$ (G) integrants. DNA fragments (B) and (D) were prepared by digesting the heterodimer plasmids of Figs. 3E and 3H with *Hind*III and *Not*I respectively. Restriction fragment (F) was the *Pvu*II digest of the plasmid 38HGBDEm^r of Fig. 1C.

Fig. 5 is a diagram of a preliminary experiment demonstrating resident plasmid integration in *S.gordonii*. Restriction sites are indicated with abbreviations as for Figs. 1 and 3. Also N=*Nco*I; and RV=*Eco*RV.

Fig. 6A,B and C show the structures of plasmids and illustrate integration of the hybrid *GBD* gene into resident plasmids harbored in *S.gordonii* cells.

Fig 6D shows in more detail the DNA sequences which recombine.

Fig 6E shows the result of agarose gel electrophoresis in which the lanes are:

M, a mixture of λDNA *Eco*RI-*Hind*III double digests and λDNA *Hind*III digest;
1, initial resident plasmid pPIOZ'18Spec^r;
2 and 3, and 4 and 5, primary and secondary transformants, respectively;
2 and 4, and 3 and 5, positive and negative control integrants, respectively.

Fig. 7 shows (on successive lines) restriction maps of the *S.mutans* GS-5 chromosomal structure containing the *gtfD* gene, the *gtfD* gene (as used for probe DNA) and the 5'- and 3'-flanking regions of the *gtfD* gene. Restriction sites shown are as described under Figs. 1, 3, and 5 above; in addition (D) denotes a non unique *Dra*I site; and Nh=*Nhe*I.

Fig 8 is a bar chart of results from Example 3.

Fig 9 illustrates a slide with an array of wells, used in Example 4.

Fig 10 shows a restriction map of the galactose oxidase gene of *Dactylium dendroides*, and the incorporation of a gene fragment into a plasmid.

Figs 11 and 12 diagramatically illustrate further manipulation to construct a plasmid containing the whole of the gene.

Figs 13 and 14 diagramatically illustrate the construction of a plasmid incorporating both the GBD gene and the galactose oxidase gene.

Fig 15 shows the results of a galactose oxidase activity assay.

Fig 16 shows the results of a glucan binding assay.

Example 1 - Preparation of Fusion Proteins Containing GBDs

As shown by the restriction maps of Fig 7, the *gtfD* gene within the chromosome of *S.mutans* GS-5 contains the gene for a glucose binding domain (GBD) within a 1.67-kb *Xba*I-*Bam*HI fragment near to its 3' terminal. [see Hondo,O., Kato,C., and Kuramitsu, H.K. (1990) Nucleotide sequence of the *Streptococcus mutans* gtfD Gene encoding the Glucosyltransferase-S Enzyme. J Gen Microbiol 136:2099-2105]

a) **Preparation of the hybrid GBD gene.** A secretion domain from the *S.mutans* GS-5 *gtfB* gene which specifies the first 38 amino acid residues from the initiator Met as the signal peptide for secretion of the GTF-I enzyme had been isolated previously (Infect Immun 61:3745).

A plasmid designated pUC18 38Hind (Fig 1A) was constructed which contains this domain (800-bp *Hind*III fragment) oriented in the opposite direction relative to the *lacZ'* gene of pUC18. The 3'portion of the *Xba*I-*Bam*HI fragment (1.67-kb) of the *gtfD* gene (5),containing the *GBD* gene (Fig.7), was then cloned into *Xba*I-*Bam*HI cleaved plasmid pUC18 38Hind. In the resulting plasmid (Fig 1B) designated 38HGBD, the reading frames of both the 3'-*Hind*III site in the signal sequence coding region and the 5'-*Xba*I site in the *GBD* gene are the same as that of the multiple cloning site (MCS) of pUC18. Consequently, the GBD molecule is expressed fused to the GTF-I protein leader sequence. For a negative control, the unique *Xba*I site was digested, filled-in and recircularized to create a plasmid in which the reading frame of the *GBD* was out of frame with the signal

sequence. The presence or absence of the GBD protein in crude extracts of *E.coli* transformants harbouring each plasmid was confirmed by Western blot analysis (data not shown).

The plasmid of Fig 1B was further modified to incorporate an Em^r gene in its unique *Bgl*II site (38HGBDEm^r, 6.3-kb, Fig.1C). This was subsequently used in transformation of *S.gordonii* as mentioned below.

b) **Integration of the hybrid *GBD* gene into the *S.gordonii* chromosome.** Attempts were made to clone a fragment containing the hybrid GBD gene into an *E.coli*-streptococcus shuttle plasmid designated pResEm749. This was unsuccessful, making it necessary to devise an alternative cloning strategy. The approach which was adopted sought to introduce the hybrid *GBD* gene directly into *S.gordonii* without utilizing *E.coli*-streptococcus shuttle plasmid.

The internal 1.5-kb *Hind*III fragment of the *S.gordonii gtfG* gene was chosen as a target site for integration of the hybrid gene into the *S.gordonii* chromosome. As shown in Fig 4, the *gtfG* gene in wild type *S.gordonii* encompasses three *Hind*III fragments. The middle one of these was chosen as the target fragment. This fragment and one adjoining *Hind*III fragment have been cloned in the known plasmid pAM5010 [Sulavik,M., Tardof,G., and Clewell, D.D. (1992) Identification of a Gene *rgg* which regulates expression of glucosyltransferase and influences the SPP Phenotype of *S. Gordonii* Challis, J Bacteriol 174, 3577-3586]. The plasmid pAM-S15 contains the 3'-end of the 1.5-kb *Hind*III fragment of the pAM5010 insert.

As an initial step, prior to the intended transformation of the S. Gordonii chromosome, it was necessary to introduce appropriate integration anchor sites into the chosen target fragment. In order to construct intermediates for this task, a novel "heterodimer" system was designed. This allows DNA fragments to be placed between the 5'- and 3'-flanking regions of a target site.

This "heterodimer" system is explained in general terms with reference to Fig.2. A plasmid referred to as the "acceptor" is shown at Fig 2A. It has a single *Pvu*II site designated in F1g 2 as site A. Initially, the target gene containing the anchor sites (Fig 2F) is cloned into the acceptor plasmid following appropriate conversion of the single *Pvu*II site, yielding the plasmid shown at Fig.2B. Next, the *Pvu*II site of another plasmid is modified by ligating a linker DNA so that the resulting "rescue" plasmid shown in Fig 2C has a site compatible with a unique site for restriction by enzyme B, which is present in the insertcloned into the acceptor plasmid. The plasmids of Figs 2B and 2C are both digested to completion with the restriction enzyme B. The resulting two DNA fragments are ligated without any phosphatase treatment and transformed into *E.coli* JM109. The plasmid DNA purified from these transformants selected on LB agar plates containing a combination of both antibiotics would be a dimer containing two identical replication regions (p15Aori) as well as two different drug resistance markers (Fig.2D). Therefore, this plasmid is designated as a "heterodimer". Finally, the DNA fragment for integration is readily prepared by digesting the heterodimer plasmid of Fig 2D with restriction enzyme A. This leaves a moiety termed pRes located between the correctly oriented 5'- and 3'- flanking regions of the target gene. "Rescued" plasmid (E) would be isolated following recircularization, and transformation into *E. coli*. The structure of the insert in the rescued plasmid (Fig.2E) has a different arrangement, 5'-B-A-B-3', compared to that of the target site, 5'-A-B-A-3'.

This procedure, just described in general terms, was used as shown by Fig 3.

To provide the appropriate integration anchor sites, two plasmids analogous to that of Fig 2B, designated pResAmpdBC (2.1-kb) and pResKmHindS-15 (3.5-kb) were constructed (Figs 3A and D, respectively). The former plasmid contains both 250-bp 5'- and 3'-flanking regions of the *gtfB* and *gtfC* genes, respectively, from *S.mutans* while the latter contains the 1.5-kb internal *Hind*II fragment of the *gtfG* gene from *S.gordonii* (18). The unique *Sca*I site present in this insert within the latter plasmid was further converted to a *Pst*I site by introducing the *Pst*I linker DNA.

The structure within the MCS of the plasmid pResAmpdBC shown in Fig 3A was:

Not-Bcl-Hind-Sph-Pst-5' end-Bgl-3' end-Pst---Bam/Bgl-Not.

Into the unique *Bgl*II site of the insert, the *Bgl*II cleaved rescue plasmid, pResEmBgl (1.6-kb, Fig.3B) was ligated. The resultant heterodimer plasmid pResAmpdBC:pResEmBgl (3.7-kb, Fig.3C) was isolated from *E.coli* JM109 transformants following selection of colonies on LB agar plates supplemented with both Amp and Em. The structure within the MCS of this plasmid would be:

Not-Bcl-Hind-Sph-Pst-5' end-Bgl:pResEmBgl:Bgl-3' end-Pst---Bam/Bgl-Not.

This heterodimer plasmid was digested with *Pst*I and a 2.1-kb fragment containing pResEmBgl flanked by both the 5'- and 3'-end fragments was gel purified. This fragment was ligated with the PstI cleaved plasmid, pResKmHindS-15P (3.5-kb, Fig.3D) and the heterodimer plasmid, pResKmHindS-15P:pResEmdBC (5.6-kb, Fig.3E) was isolated. Digestion of this heterodimer plasmid with *Hind*III leads to the fragment shown at Fig 4B.

S.gordonii was transformed using the *Hind*III digested plasmid. The common regions between the chromosome and DNA fragments where double cross over can occur are the 5'- and 3'- fragments of the pAM-S15 insert shown with thick lines in Figs 4A and 4B. *S.gordonii* primary integrants (Em^r, GTF^-, Fig.4C) which contain an integration anchor site replacing the *Sca*I site of the *gtfG* gene were isolated following transformation.

The 1.2-kb *Hind*III-*Bam*HI fragment (containing the 3'-portion of the *gtfD* gene, Fig.7) was introduced into the HindIII-BglII cleaved plasmid pResAmpdBC (Fig 3A) to yield pResAmp3'GBDdC (3.0-kb, Fig.3F). The structure of the insert in this plasmid was:

Not-Bcl-Hind-3' GBD-Bam/Bgl-3' end-Pst---Bam/Bgl-Not.

Subsequently, pResSpecHind (1.8-kb, Fig.3G) was cloned into the unique *Hind*III site of pResAmp3'GBDdC and the heterodimer pResAmp3'GBDdC:pResSpecHind (4.8-kb, Fig.3H) was isolated. The structure around the insert is:

Not-Bcl-Hind:pResSpecHind:Hind-3' GBD-Bam/Bgl-3' end-Pst---Bam/Gbl-Not.

*Not*I digestion of this plasmid leads to the fragment illustrated at Fig 4D.

The *S.gordonii* primary integrants containing the 5'- and 3'-flanking regions of the *gtfB* and *gtfC* genes (Fig.4C) were transformed with this heterodimer plasmid following *Not*I digestion (Fig.3D) and secondary integrants (Em^s, $Spec^r$, 3'GBD, Fig.4E) were isolated. The common regions between the chromosome and DNA fragments where double cross over can occur are p15Aori and the 3'-end of the *S.mutans gtf*C gene.
Finally, this integrant was transformed with the plasmid $38HGBDEm^r$ (Fig.1C) following *Pvu*II digestion to yield the fragment shown at Fig 4D. The common regions between the chromosome and DNA fragments where double cross over can occur are the 5'-end of the *S.mutans gtf*B gene and 3'-end of the *GBD* gene. Following transformation $Spec^s$, Em^r, GBD^+ transformants were isolated (Fig.4G).

The corresponding GBD^- transformants were constructed in the same way, using the negative control hybrid gene created, as described above, by change at the *Xba*I site in the 38HGBD plasmid shown at Fig1B.

c) **Resident plasmid integration.** A preliminary experiment was carried out to been mentioned above, the hybrid *GBD* gene could not be cloned into the shuttle plasmid pResEm749 in *E.coli*. Since *S.gordonii* is a strain, chromosomal integration could be readily accomplished with an appropriate linear DNA fragment following transformation. This approach was examined by determining whether a DNA fragment residing on a plasmid replicating in *S.gordonii* (which is a recombination proficient strain) could be replaced following integration events. This is illustrated by Fig.5. Em^r and $Spec^r$ genes

were (separately) cloned into the *Bam*HI site of plasmid KmOZ'18, a pUC-type Km^r vector (Infect Immun 61:3745). Since the Km^r gene of this plasmid is flanked by *Xho*I sites, this drug resistance gene was eliminated by *Xho*I digestion and a *Bgl*II linker was introduced following a filling-in reaction. In the shuttle plasmid pResEm749, the *Cla*I and one of the *Sca*I sites were converted to *Bam*HI sites following several DNA manipulations. The basic replicon from pVA380-I (2.5-kb *Bam*HI fragment, ref.7) active in streptococci was isolated from this pResEm749 derivative and gel purified. It was used to construct *E.coli*-streptococcus shuttle plasmids pPIOZ'18Em^r (4.9-kb) and pPIOZ'18$Spec^r$ (5.1-kb) (pPI:resident Plasmid Integration). *S.gordonii* competent cells were then transformed with each shuttle plasmid and Em^r and $Spec^r$ strains were isolated. An Em^r strain harbouring pPIOZ'18Em^r was next transformed with the *Bgl*II linearized plasmid dKmOZ'18$Spec^r$. These have two regions (pUCori and downstream of *lacZ*') in common and double crossover can occur via these two homologous regions. As a result, an Em^r gene was replaced with a $Spec^r$ gene following selection of *S.gordonii* transformants on TSB containing Spec. Plasmid DNA was isolated from the $Spec^r$ transformants which was indistinguishable from the pPIOZ'18$Spec^r$ shuttle plasmid previously constructed using *E.coli* cells. Similar results were obtained when the $Spec^r$ *S.gordonii* was transformed with the linear dKmOZ'18Em^r DNA (as illustrated at the right hand side of Fig5).

These observations indicated that integration events following transformation could occur not only on the chromosome but also within resident plasmids in *S.gordonii* cells. Therefore, certain plasmid structures which are unstable in *E.coli* cells might be constructed directly in a *S.gordonii* system without prior ligation of component DNA fragments. To put this novel strategy into effect, *S.gordonii* $Spec^r$ transformants harbouring pPIOZ'18$Spec^r$ (as shown in Fig5 and also Fig6A) were transformed with *Eco*O109 linearized 38HGBDEm^r (Fig1C also Fig6B) which contained the hybrid *GBD* gene. Resident plasmid pPIOZ'18$Spec^r$ and *Eco*O109 linearized 38HGBDEm^r share two homologous domains (pUCori and downstream of *lacZ'*) and the hybrid GBD gene was integrated following recombination. Analysis of plasmid DNA from primary Em^r transformants indicated the presence of two types of plasmids within a single transformant:

initial and integrant plasmids lanes 2 and 3). Secondary Em^r isolates transformed using these plasmids indicated the presence of only one plasmid, pPI38HGBDEm^r (7.5-kb, lanes 4 and 5). Transformation of *E.coli* JM109 with the integrant plasmid from the latter transformants yielded no Em^r colonies again indicating that this plasmid cannot be maintained in *E.coli* cells.

d) **Preparation of GBD fusion proteins.** Following growth of *S.gordonii* transformants (containing either a plasmid or chromosomal copy of the GBD-fusion gene), the culture supernatant fluids were concentrated 50 times by acetone precipitation and assayed for the presence of GBD. Western blot analysis using anti GTF-S antibody as described previously (*Gene* 69:101-109) confirmed secretion of the protein bands of the expected size (60 kd) as well as smaller degradation products.

e) **Expression of GBD hybrid genes in** *E. coli.* GBD can be expressed in *E. coli* as a fusion protein containing peptides from other proteins.

A *Xba*I fragment from the *gtf*D gene was inserted in frame into the plasmid pGD103X. This then expressed a fusion of the GBD with the first thirteen amino acids of ß-galactosidase. The fusion protein readily binds glucans as determined by: binding of biotinylated-dextran by the fusion protein or after attachment of the fusion protein to dextran-Sepharose beads followed by elution and detection on Western blots with anti-GTF-S sera. This strategy also incorporates a cysteine residue into the fusion protein derived from the ß-galactosidase peptide. This allows for the covalent attachment of biotin or other detection molecules to the GBD.

In addition, the GBD has been fused to the Tag peptide of the pTOPE system (Novagen, Madison, Wisconsin) for expression in *E. coli*. The resultant fusion protein is naturally biotinylated in *E. coli* and can be readily purified following absorption to avidin columns. Following elution of the fusion protein with biotin the GBD can be released from the fusion protein following Factor Xa cleavage and passage of the mixture through a second

avidin column.

GBD-β galactosidase peptide fusion proteins were purified from transformed *E.coli* by applying cell extracts to dextran-Sepharose columns and eluting with a gradient of guanidine HCl in 10 mMol acetate buffer (pH=6.0). This was followed by dialysis into buffer devoid of guanidine HCl.

Example 2 - Binding of 6BD fusion proteins to dextrans

a) **Binding to biotinylated-dextran.** An enzyme linked immunoassay (ELISA) for measuring dextran binding activity was devised utilizing biotinylated-dextran (Pharmacia). Briefly:

Protein samples (50 uL of culture supernatant from *S. Gordonii* transformants) were added to wells in microtiter plates and incubated for 18 h at 4°C. The supernatant fluids were discarded and the absorbed proteins washed three times with water. Each well was then filled with 200 uL of blocking buffer (0.5% BSA in acetate buffer, pH 6) and incubated for one hour at 37°C and washed as described above. The wells can then be filled with various concentrations of biotinylated-dextran (Pharmacia) and incubated for 10 min at room temperature. Each well was then washed as described above with buffer. Streptavidin-peroxidase was then added and incubated for 5 mins at RT. The contents of each well was removed and the absorbed material washed as described above. The colour reaction was then developed by adding the peroxidase substrate and incubated for 10-30 min and the reaction terminated by adding 100 uL of 3N sulfuric acid. The absorbance of each solution was then measured at 410 nm in an ELISA plate reader.

Using this assay, culture supernatant fluids were compared from a *S.gordonii* strain either harbouring the GBD fusion chromosomal integrant and a control strain with no integrated gene for the hybrid protein. The results in Table 1 clearly show that the strain harbouring the GBD fusion protein gene secreted a protein which binds to biotinylated-dextran in a

dose dependent manner. In contrast, the negative control strain does not produce this activity.

Table 1 Glucan binding by S.gordonii transformants

	A_{410}			
Biotin-dextran (ug/ml)	0	1	10	100
S.gordonii dXba (neg.control)	.047	.053	.049	.050
S.gordonii 38HGBD (integrant)	.051	.076	.101	.201

Example 3 - Binding of GBD-biotin conjugates to bacteria

a) **Preparation of glucan-binding domain-biotin conjugates.** In order to demonstrate that GBDs can be used to target other molecules to dental plaque, conjugates of GBD and biotin were produced and tested for binding to glucan-coated bacteria, and for targeting to plaque. To produce GBD-biotin the amino acid cysteine can be engineered genetically into the structure of the protein (GBD is normally devoid of cysteine) at either the C or N terminus. This can be done by adding the appropriate codon to the sequence of the GBD gene or by adding cysteine codons in the peptide sequences fused to GBD. In the experiments described here, cysteine was added to GBD as part of the sequence of the β-galactosidase peptide in the GBD fusion produced in E.coli.

Biotin was attached to the GBD fusion protein by a previously described method (Anal. Biochem.149:529; 1985). To do this, a solution of the GBD (purified from *E.coli* clone extracts on dextran-Sepharose columns) was mixed with 0.3 mg of biotin-maleimide (Sigma). The solution was incubated for 5 hr at 37°C and dialysed against phosphate-buffered saline for 18 hr to remove unreacted biotin. The resultant biotinylated-GBD could be used to quantitate binding of the complex to bacterial cells or dextran by mixing the GBD with beads or cells and filtering the mixtures on Ultra-free MC filters (Millipore). The samples could be washed directly on the filters and incubated with streptavidin-

peroxidase to quantitate the amount of GBD bound to the cells or resins following determination of the absorbance of the coloured conjugates in an ELISA reader.

b) **Measurement of binding**

To measure binding of GBD-biotin to glucans on bacterial surfaces, *Streptococcus mutans* GS-5, a producer of glucans from dietary sucrose, was grown in Todd Hewitt Broth with either glucose or sucrose as carbohydrate source. Growth of *S.mutans* in sucrose results in the production of cell associated glucans (*Microbiol. Rev.* 44:331-384). In contrast, growth of the bacteria in glucose results in no glucan production.

After growth in suspension at 37°C, the bacteria were washed with buffer and resuspended in the absence or presence of GBD-biotin. The bacteria were washed again with buffer and incubated with strepavidin peroxidase (Gibco/BRL) dissolved in 0.1 M Tris HCl pH=7.5 containing 0.1 M NaCl, 2 mM $MgCl_2$, and 0.05% Triton α-100. After washing, the cells were incubated with colour reagent solution (1 mg/ml o-phenylenediamine HCl and 0.012% hydrogen peroxide in citrate buffer; pH=4.5). The cell binding by GBD-biotin was thus measured as absorbance at 410 nm following colour development. The results are shown in Fig. 8, and it is clear that incubation of *S.mutans* in sucrose-medium results in enhanced binding of GBD-biotin due to production of the natural binding site for GBD, bacterial glucan. Glucose grown cells did not provide significant amounts of binding sites for GBD-biotin (ie. they did not produce glucan).

<u>**Example 4 - Binding of GBD-biotin to human plaque**</u>

This example demonstrates that GBD will bind to plaque, and this binding may be detected by an anti-GTF antibody, which in turn may be detected by an anti-species antibody conjugated to a fluorescent marker. Binding can then be visualised on a slide using a fluorescent microscope.

Method

Sample Preparation

Human plaque was obtained from volunteers who had used 10 ml of a 5% sucrose rinse three times on the day before plaque sampling. They had also not brushed their teeth for 24 hours prior to plaque sampling. The plaque was frozen immediately after collection and stored at -20°C until used. The plaque was intensively sonicated (in 1 second cycles: 0.5 seconds on and 0.5 seconds off) for 30 seconds in Ringer's solution containing 2% formalin. Aliquots of 10 μl of plaque suspension, diluted to 1 mg/ml, were pipetted into glass slides bearing 8 sample "wells" as shown by Fig 9. The wells were allowed to air dry, then briefly flamed to fix.

Sample Treatment

The slides were blocked to reduce non-specific binding by immersion in phosphate-buffered saline pH 7.2 containing 1% BSA and 0.05% Tween 20 (blocking buffer) for 20 minutes. The solution was rinsed off by immersing in two successive Ringer's solution baths. Subsequently, solutions containing other reagents were added in this buffer using the same procedure. GBD at 20 μg/ml was added, followed by rabbit polyclonal anti-GTF-S antibody at dilutions from x 50 to x 2000 as indicated in Fig. 9. A GBD-free well and a blank well were used as controls.

Detection of Bound GBD

The fluorescence of the prepared slide was examined using a mercury lamp and epi-illumination. Photographs of representative areas of the slides were taken using HS-400 Ektachrome film.

Results

The photographs of the fluorescence of plaque samples after treatment showed that intense fluorescence was present in these samples. There was a strong dependence of this fluorescence on the GBD concentration used, showing that GBD binds to plaque, even in the presence of a blocking protein.

Conclusions

These experiments demonstrate that GBD binds strongly and in considerable amounts to plaque, in a concentration-dependent manner. However, some fluorescence was still present on the GBD-free control. This is most likely to be due to binding of anti-GTF-S antibody to GTF naturally present in plaque. These results are the first evidence that GBD, devoid of the remainder of the parent GTF molecule, retains the ability to bind to glucans and target to human plaque.

Example 5

An experiment was carried out to demonstrate binding to biofilms, analogues of plaque on teeth. The procedure was as follows:

(a) An overnight culture of *S.mutans* was added to cells of a sterile microtitre plate, incubated for 1 hour, then poured off.

(b) After a rinse with phosphate buffered saline (PBS) the plate was incubated for 2½ hours with a 1% solution of sucrose in PBS. In both cases the bacteria were expected to form a film, but with sucrose the film will include glucan.

© The plate was incubated for 30 minutes with a solution containing G5D produced according to Example 1C, then washed 3 times with PBS.

(d) The plate was incubated for 30 minutes with a suspension of rabbit polyclonal antibodies to GTF, then rinsed with PBS.

(e) The plate was incubated for 30 minutes with a suspension of a conjugate consisting of goat anti-rabbit polyclonal antibodies attached to horse radish peroxidase (HRP) enzyme, then washed 10 times with distilled deionised water.

If the films on the on the cell contained glucan, this should be anchoring on the plate some of the GBD, and through this the antibodies of steps (d) and (e). The resulting complex would be

substrate: GBD: rabbit anti GTF: anti-rabbit-HRP.

The cells were supplied with substrates which can be converted by HRP enzyme to generate a blue colour. The rate of change of absorbance at 630 nm was measured.

If the GBD, or anti GTF antibody was omitted, there was very little colour formation. If the whole system was complete, colour formation was six times greater. If glucose was used in place of sucrose in step (b), there was very little colour formation.

This indicates that GBD was binding successfully to the film (a model of plaque) made by *S.mutans* in the presence of sucrose. With glucose in place of sucrose there was no glucan formation and nothing for the GBD to bind to, as was demonstrated by comparative experiments.

If dextran, at a concentration of 1 mg/ml or more was included in the GBD solution used at step (c), it provided an alternative site for binding by GBD. It suppressed colour formation, indicating that GBD had bound to the dextran and subsequently been washed away with it.

Example 6

The galactose oxidase gene from the fungus *Dactylium dendroides* was contained in the plasmid pGAO [McPherson et al (1992) J. Biol. Chem. **267**, 8146-8152]. The *BamH*I-*EcoR*I fragment shown enlarged in Fig 10 and containing a portion of the leader sequence was cloned into the plasmid pUC118 to produce the plasmid pUCGAOEB (Fig11). A *Dde*I fragment was then taken from this plasmid, treated with Klenow fragment to give it

blunt ends, and inserted at the *SmaI* site in a plasmid pUC118Nar(W) which has an inactived lacZ gene. The resulting plasmid pO5 contains the fragment of the galactose oxidase gene under control of the lacZ promoter.

A *Nar*I-*Xba*I fragment was next taken from the galactose oxidase gene and inserted between corresponding sites in plasmid pO5, as shown by Fig 12, thus constructing plasmid pR3 which contains the complete galactose oxidase gene under control of the lacZ promoter. This plasmid was used to express galactose oxidase in *E.coli.*

A *Dde*I fragment from the 3' end of the galactose oxidase gene was treated with Klenow fragment to give it blunt ends and then inserted into the *Hinc*II site in plasmid pUC119, as shown by Fig 13, leading to plasmid pP4. The GBD gene constructed in part (a) of Example 1 was cut, as an *Xba*I-*EcoR*I fragment, from a plasmid analogous to that of Fig1B. It was inserted into plasmid pP4 thus creating plasmid pS2 which contained the GBD gene fused to a sequence from the 3' end of the galactose oxidase gene. The GBD gene fused to the 3' terminus of the galactose oxidase gene was then cut out as a *BamH*I-*Eag*I fragment and , as shown by Fig 14, inserted into plasmid pR3 which had been cut with *Bcl*I and *Eag*I. The result was plasmid pU4 which was used in *E.coli* to express a fusion protein consisting of the glucan binding domain fused to galactose oxidase.

E.coli strains containing the plasmids pS2, pR3 and pU4 respectively were cultured and the culture supernatants were assayed for galactose oxidase activity. Controls were provided by solutions containing various concentrations of galactose oxidase and a solution containing buffer only. The results are shown in Fig 15 from which it can be seen that the fusion protein expressed by *E.coli* cells with plasmid pU4 possessed galactose oxidase activity which was greater than the activity of the control with 10ug/ml of galactose oxidase.

These *E.coli* strains were also assayed for glucan binding activity by the procedure of Example 2.

These results show that a fusion of galactose oxidase and glucan binding domain is able to exhibit the enzyme function and also to bind to glucans.

CLAIMS

1. A polypeptide with specific binding affinity for glucan, covalently chemically bound to a further material which is not a glycosyltransferase enzyme.

2. A polypeptide according to claim 1 wherein the further material is effective to inhibit the formation of dental plaque.

3. A polypeptide according to claim 2 wherein the further material is an antimicrobial agent.

4. A polypeptide according to claim 2 wherein the further material is active against the staining of teeth.

5. A polypeptide according to claim 2 wherein the further material is selected from the group consisting of antibodies, antibody fragments, histatins, lactoferrin, defensins, magainins, cecropins, cationic antibacteriocins, bacteriocins, triclosan, chlorhexidine, quaternary ammonium compounds, chloroxylenol, chloroxyethanol, thymol and chelating agents for zinc, tin or copper ions.

6. A polypeptide according to claim 2 wherein the further material is selected from the group consisting of biotin and avidin.

7. A polypeptide according to claim 2 wherein the further material is a second polypeptide, connected to the first said polypeptide through a peptide bond.

8. A polypeptide according to claim 7 wherein the second polypeptide is an enzyme

9. A polypeptide according to claim 8 wherein the enzyme is an oxidase or a peroxidase

10. A polypeptide according to claim 1 wherein the polypeptide is a clone of a glucan binding domain of glycosyltransferase.

11. A composition for topical application in the oral cavity, comprising a polypeptide as defined in claim 1, in a carrier vehicle which is acceptable for use in the mouth

12. A composition according to claim 1 containing from 0.01% to 1% by weight of the polypeptide.

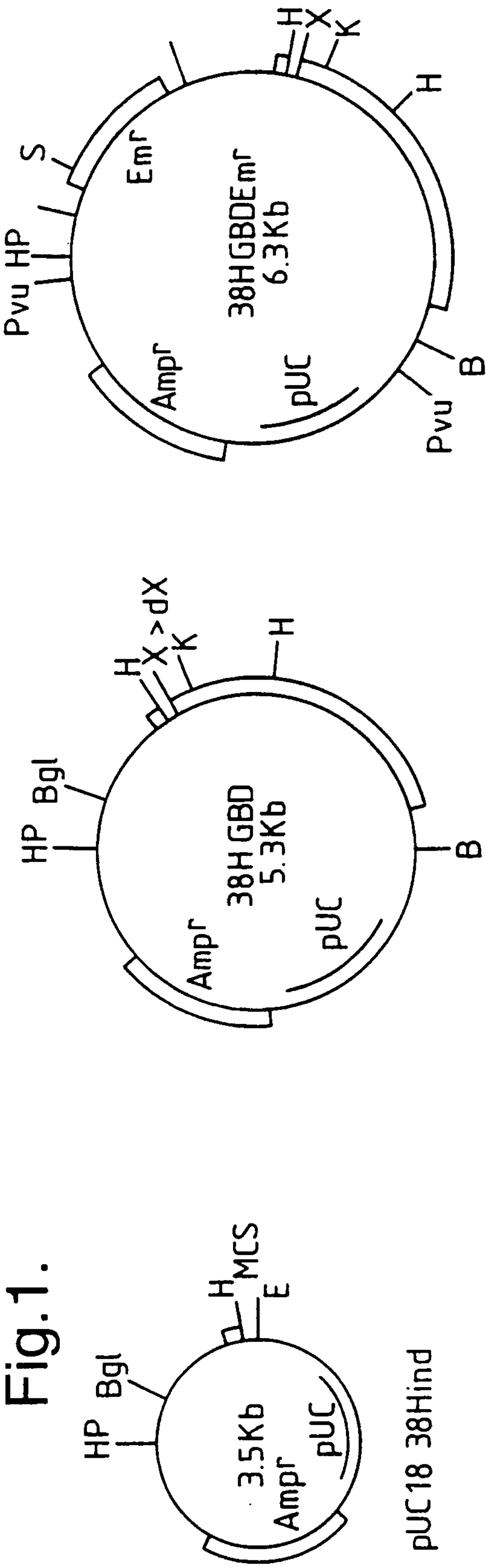
13. A product comprising a pair of cooperating compositions for topical application in the oral cavity,

the first composition comprising a polypeptide according to claim 1, covalently chemically bound to a target material which is not a glycosyltransferase enzyme;

the second composition comprising a an agent which is effective against dental plaque or tooth stain, covalently chemically bound to a material with specific binding affinity for the said target material, in a carrier vehicle which is acceptable for use in the mouth.

14. A method of inhibiting dental plaque, comprising topical application of a polypeptide as defined in claim 1 in the mouth.

Fig.1.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Bgl
H
MCS
E
3.5Kb
Ampr
pUC
pUC18 38Hind
38H GBD
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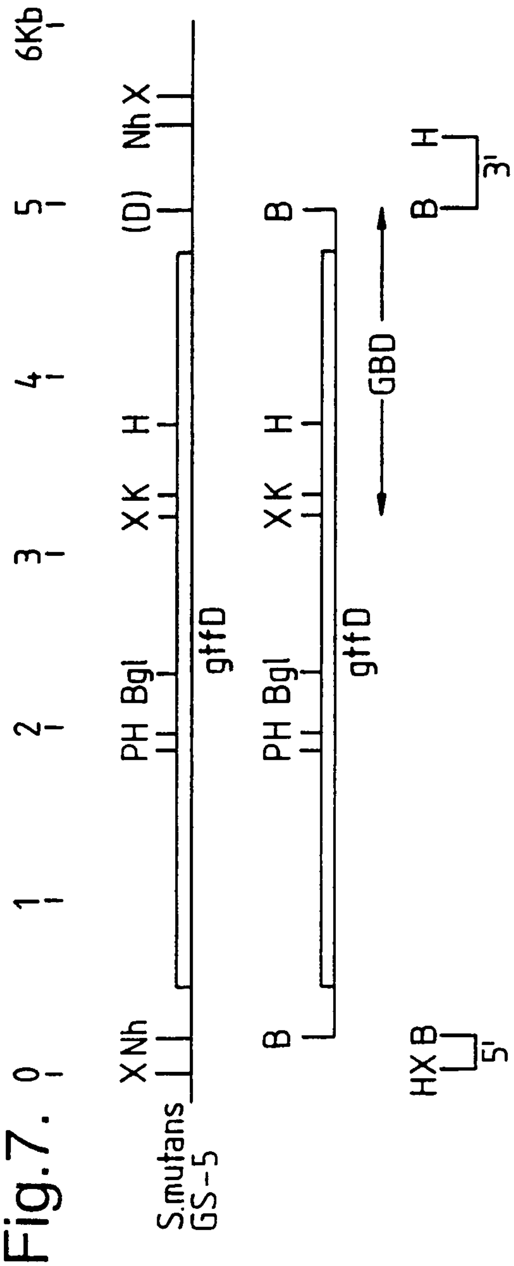
X >dX
K
B
38H GBDEmr
6.3Kb
Emr
S
Pvu
HP
X

Fig.7.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3
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S.mutans
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PH Bgl
X K
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(D)
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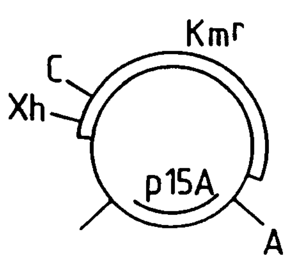
gtfD
B
GBD
HX B
5'
3'

Fig.2.

Fig.2A.



Acceptor

Fig.2B.

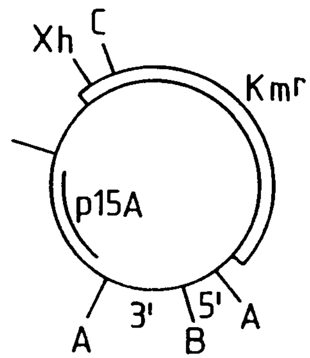
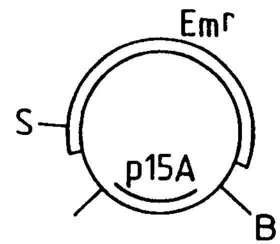
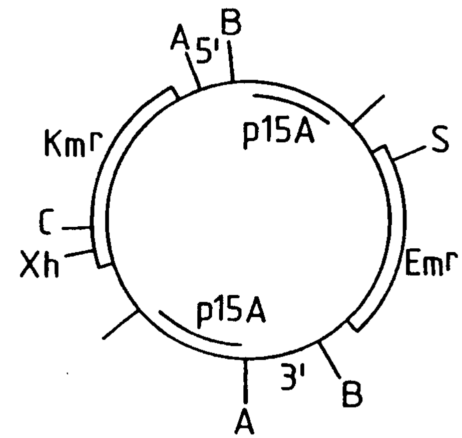


Fig.2C.



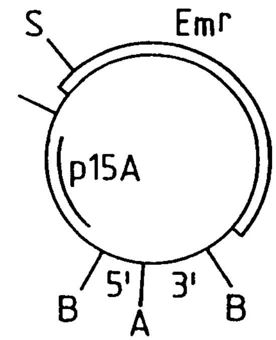
Rescue

Fig.2D.



Heterodimer

Fig.2E.



Rescued

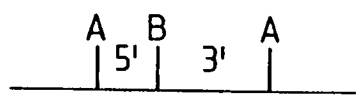


Fig.2F.

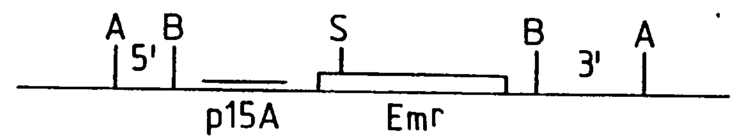
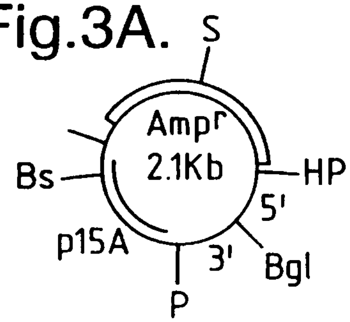


Fig.2G.

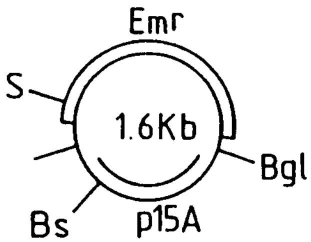
Fig.3.

Fig.3A.



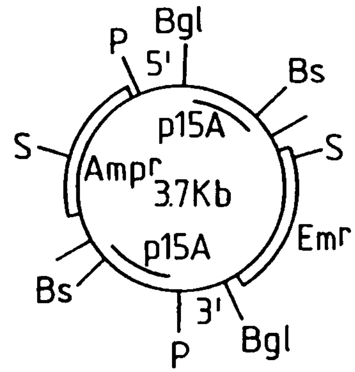
pResAmpdBC

Fig.3B.



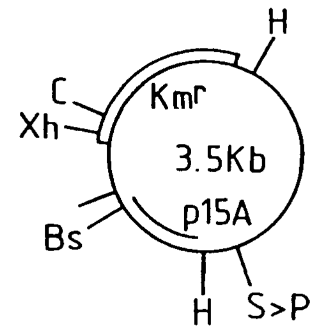
pResEmBgl

Fig.3C.



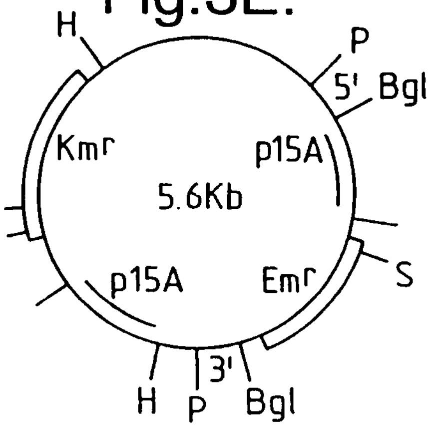
pResAmpdBC:
pReEmBgl

Fig.3D.



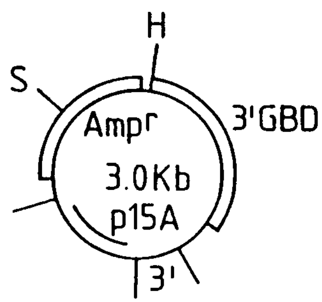
pResKmHindS-15

Fig.3E.



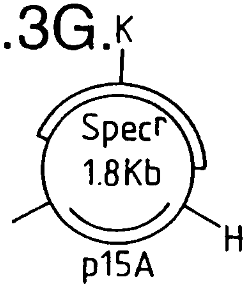
pResKmHindS-15P:
pResEmdBC

Fig.3F.



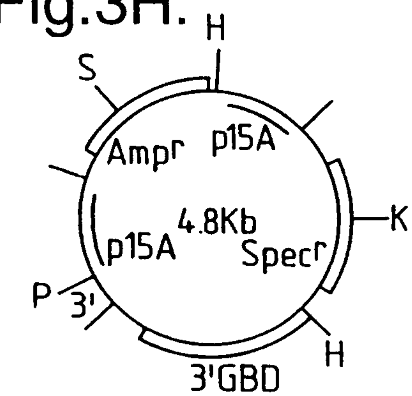
pResAmp3'GBDdC

Fig.3G.



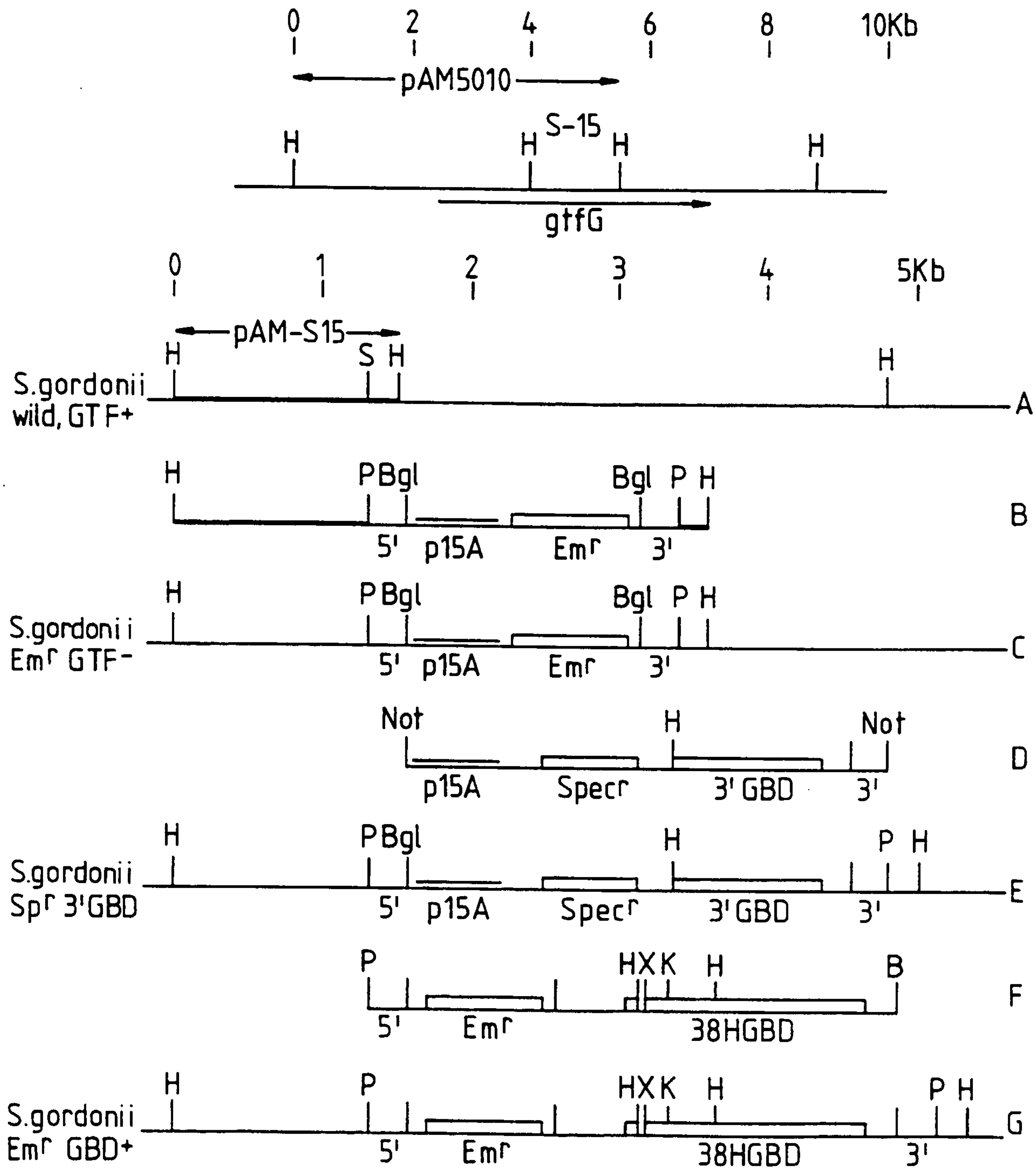
pResSpecHind

Fig.3H.



pResAmp3'GBDdC:
pResSpecHind

Fig.4.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H
H
H
gtfG
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3
4
5Kb
pAM-S15
H
S H
H
S.gordonii
wild, GTF+
A
H
PBgl
Bgl P H
5'
p15A
Emr
3'
B
H
PBgl
Bgl P H
S.gordonii
Emr GTF-
5'
p15A
Emr
3'
C
Not
H
Not
p15A
Specr
3' GBD
3'
D
H
PBgl
H
P H
S.gordonii
Spr 3'GBD
5'
p15A
Specr
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E
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HXK
H
B
5'
Emr
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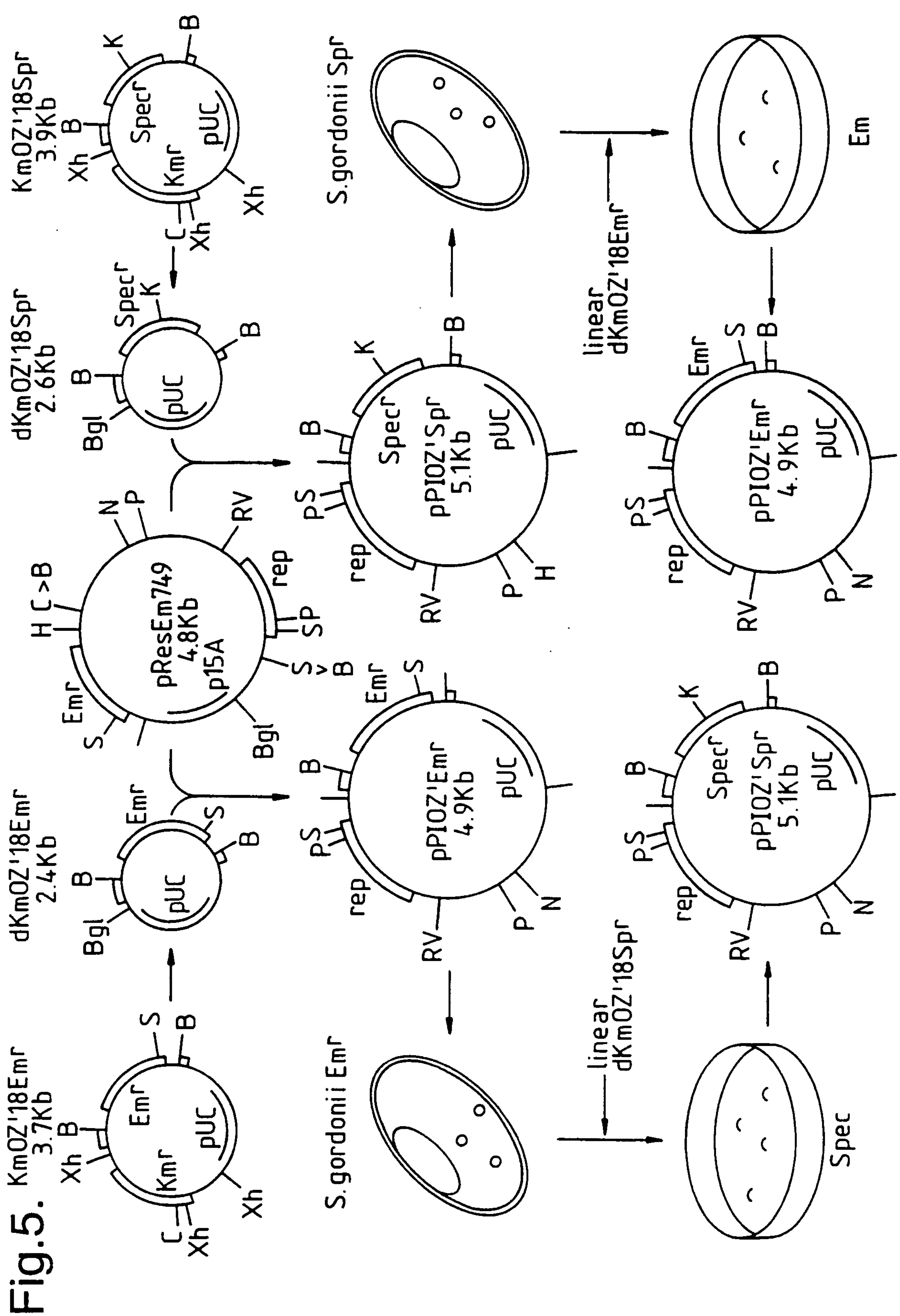
5'
Emr
38HGBD
3'
G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2.6Kb
KmOZ'18Spr
3.9Kb
pPIOZ'Emr
4.9Kb
pPIOZ'Spr
5.1Kb
S.gordonii Emr
S.gordonii Spr
linear
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linear
dKmOZ'18Emr
Spec
Em

Fig.5.

Fig.6A.

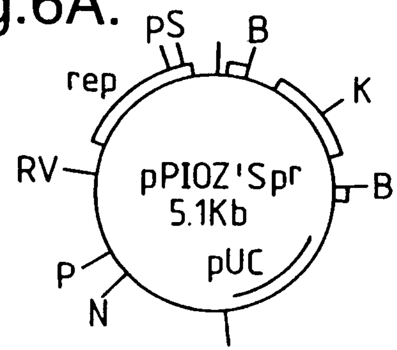


Fig.6B.

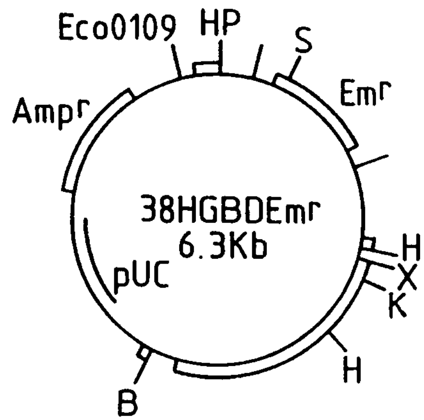


Fig.6C.

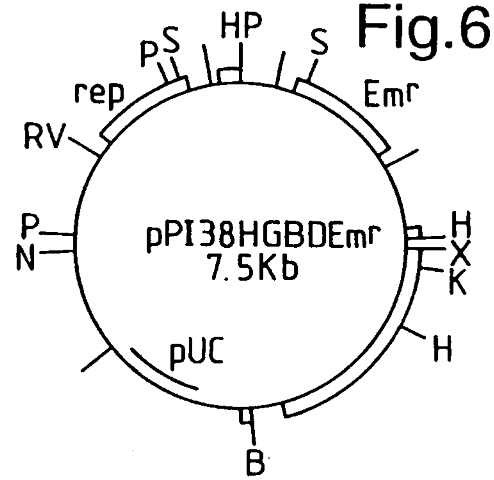


Fig.6D.

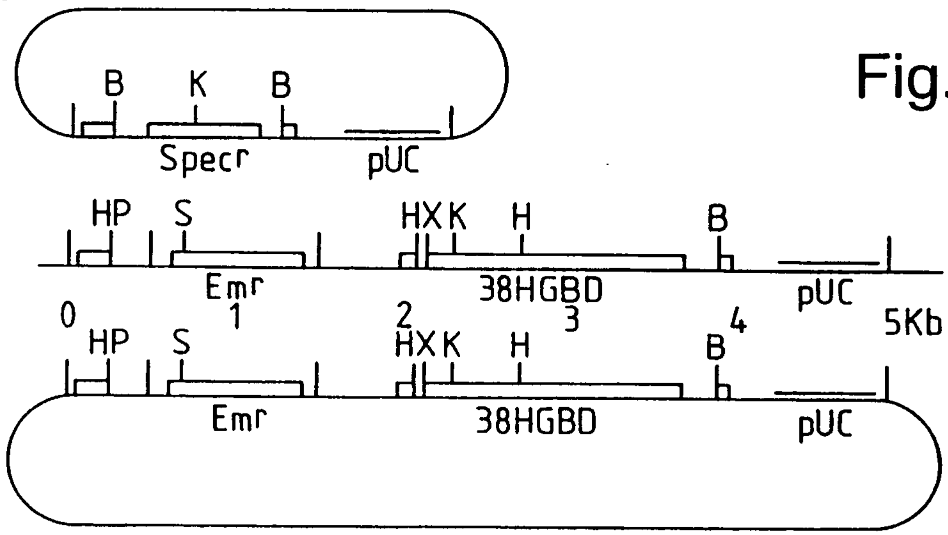


Fig.6E.

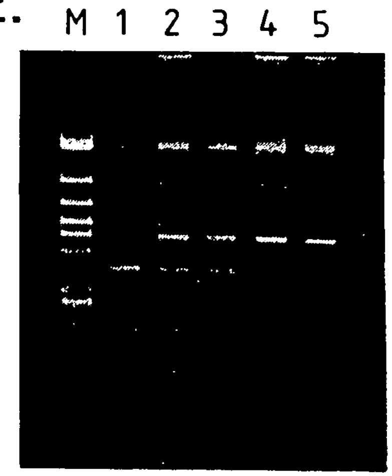


Fig.8.

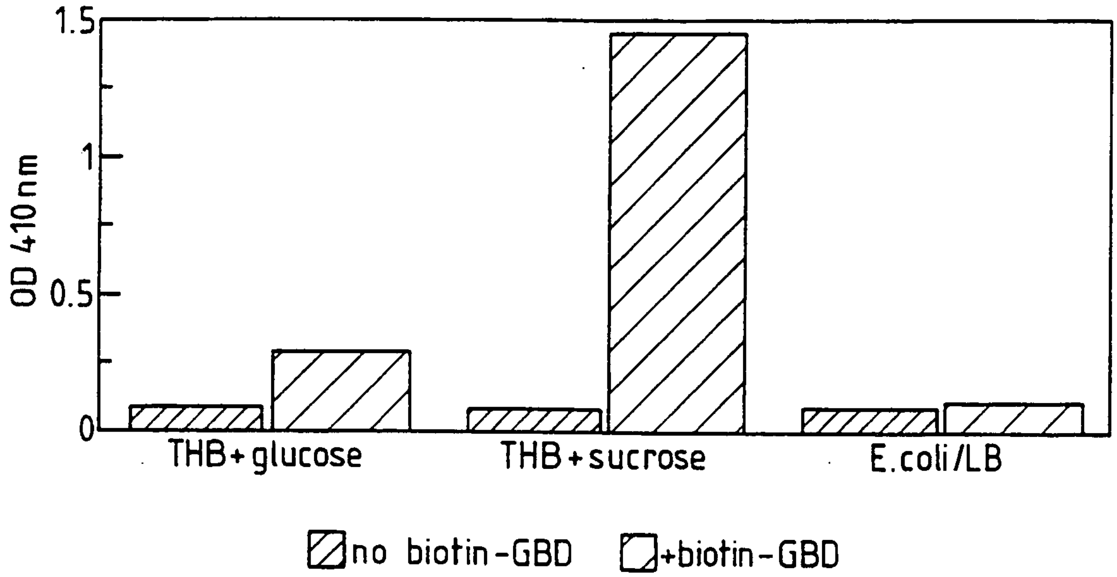

1.5
1
0.5
0
OD 410 nm
THB+glucose
THB+sucrose
E.coli/LB
no biotin-GBD
+biotin-GBD

Fig.9.

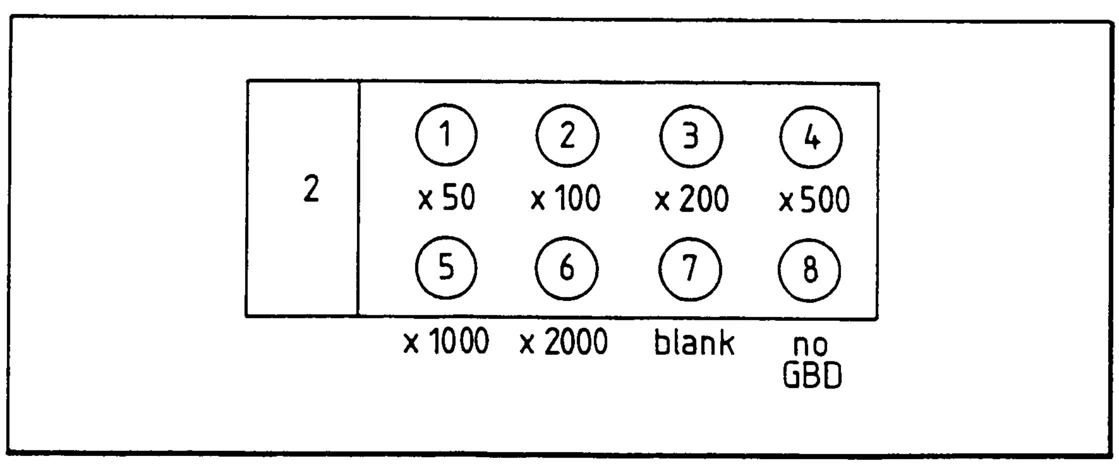

2
1
2
3
4
x 50
x 100
x 200
x 500
5
6
7
8
x 1000
x 2000
blank
no GBD

Fig.10.

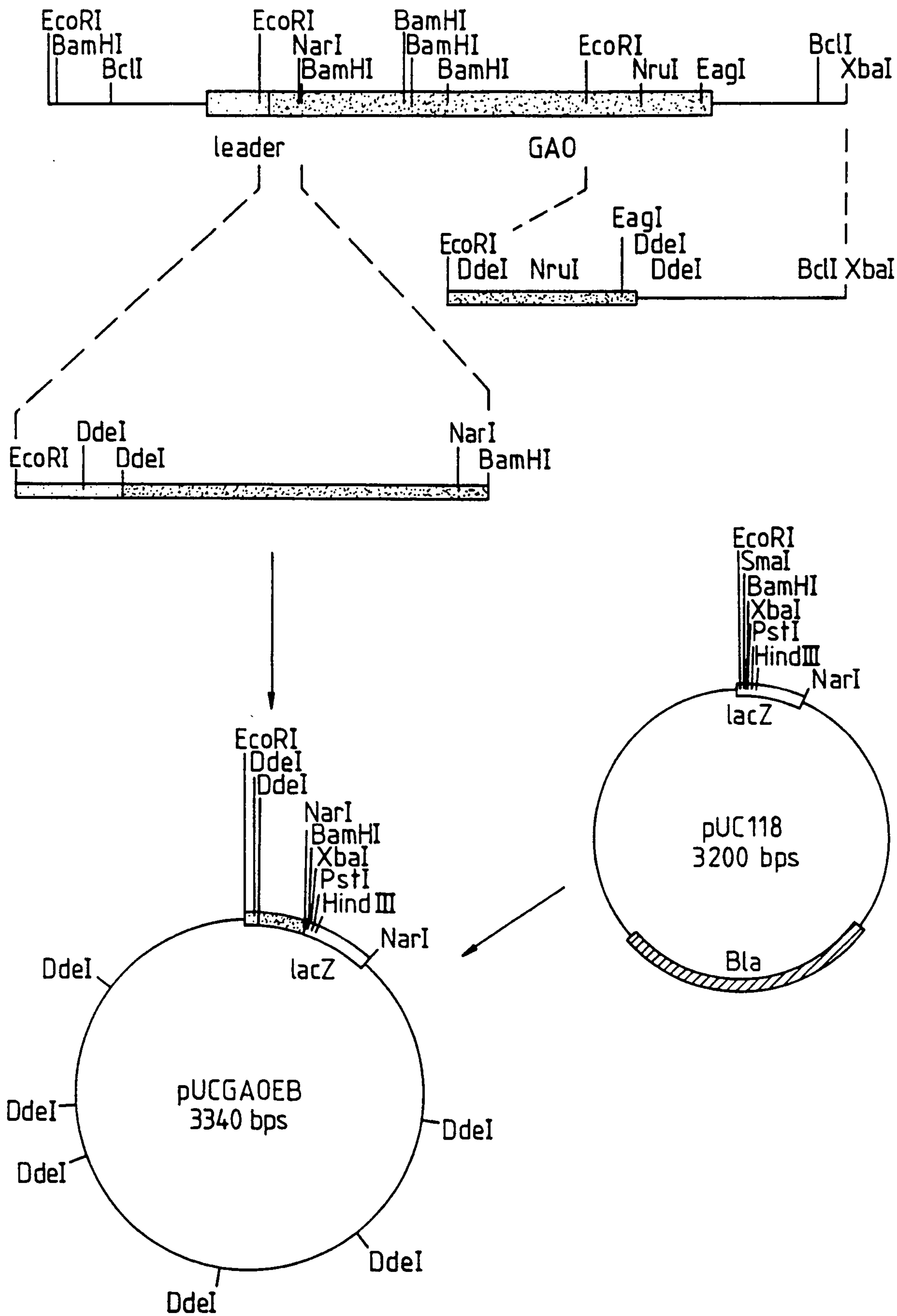

EcoRI
BamHI
BclI
EcoRI
NarI
BamHI
BamHI
BamHI
BamHI
EcoRI
NruI
EagI
BclI
XbaI
leader
GAO
EcoRI
DdeI
NruI
EagI
DdeI
DdeI
BclI XbaI
DdeI
EcoRI
DdeI
NarI
BamHI
EcoRI
SmaI
BamHI
XbaI
PstI
HindIII
NarI
lacZ
pUC118
3200 bps
Bla
EcoRI
DdeI
DdeI
NarI
BamHI
XbaI
PstI
Hind III
NarI
lacZ
DdeI
pUCGAOEB
3340 bps
DdeI
DdeI
DdeI
DdeI
DdeI

Fig.11.

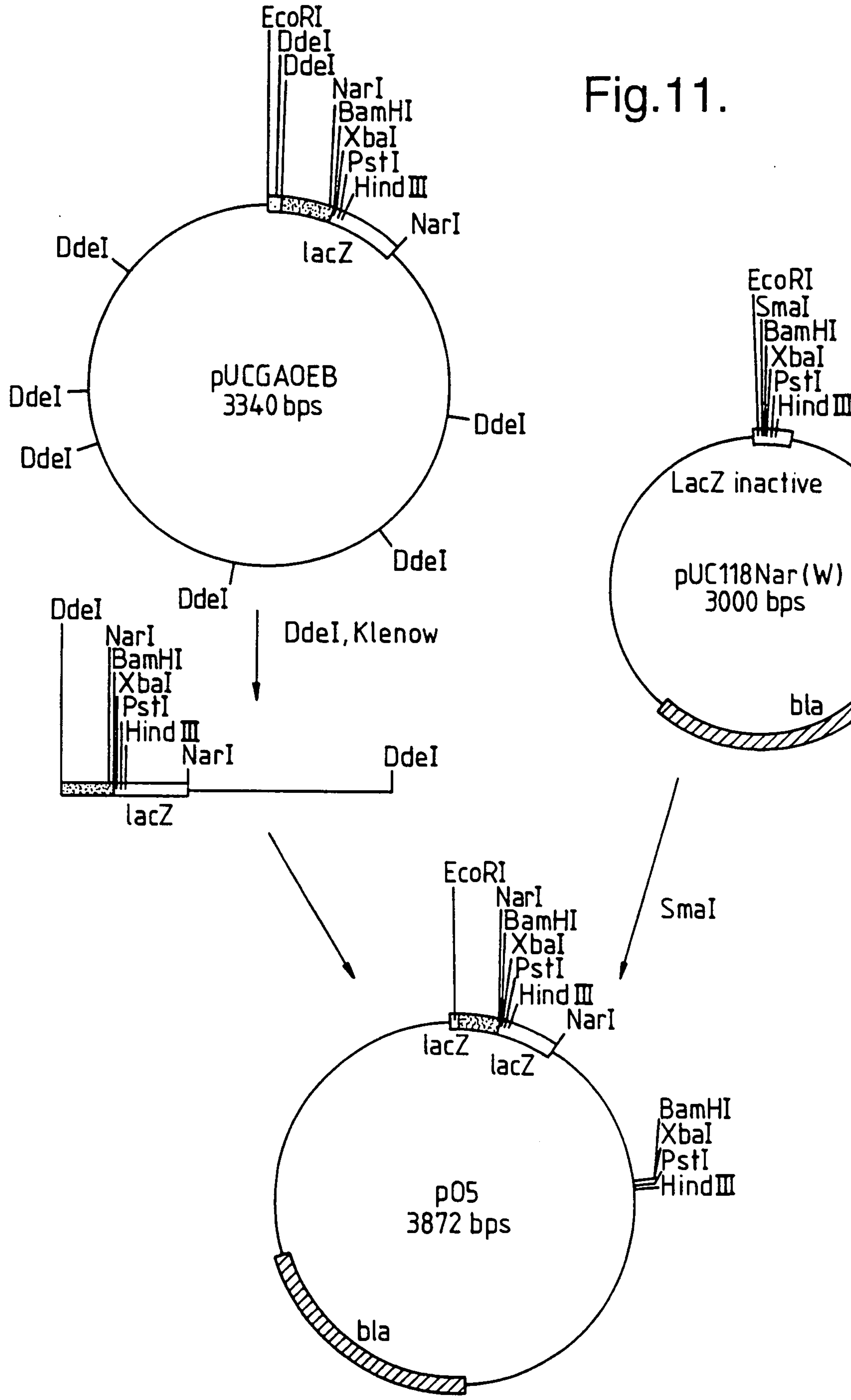
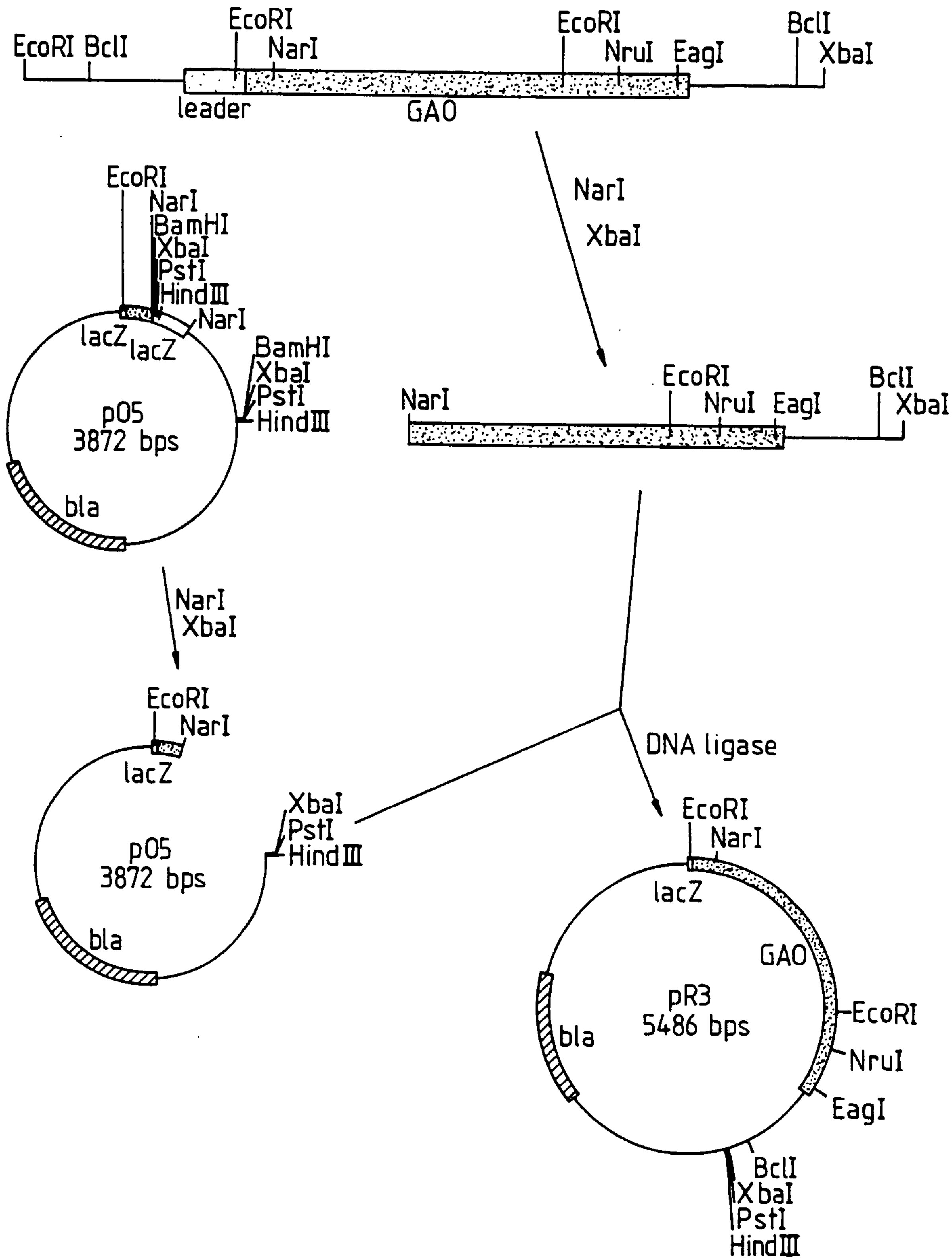

EcoRI
DdeI
DdeI
NarI
BamHI
XbaI
PstI
Hind III
NarI
lacZ
DdeI
DdeI
DdeI
DdeI
DdeI
DdeI
pUCGAOEB
3340 bps
DdeI, Klenow
DdeI
NarI
BamHI
XbaI
PstI
Hind III
NarI
DdeI
lacZ
EcoRI
SmaI
BamHI
XbaI
PstI
Hind III
LacZ inactive
pUC118Nar(W)
3000 bps
bla
SmaI
EcoRI
NarI
BamHI
XbaI
PstI
Hind III
NarI
lacZ
lacZ
BamHI
XbaI
PstI
HindIII
pOS
3872 bps
bla

Fig.12.



EcoRI
BclI
EcoRI
NarI
EcoRI
NruI
EagI
BclI
XbaI
leader
GAO
NarI
XbaI
EcoRI
NarI
BamHI
XbaI
PstI
HindIII
NarI
lacZ
lacZ
BamHI
XbaI
PstI
HindIII
pO5
3872 bps
bla
NarI
EcoRI
NruI
EagI
BclI
XbaI
NarI
XbaI
EcoRI
NarI
lacZ
XbaI
PstI
HindIII
pO5
3872 bps
bla
DNA ligase
EcoRI
NarI
lacZ
GAO
pR3
5486 bps
bla
EcoRI
NruI
EagI
BclI
XbaI
PstI
HindIII

Fig.13.

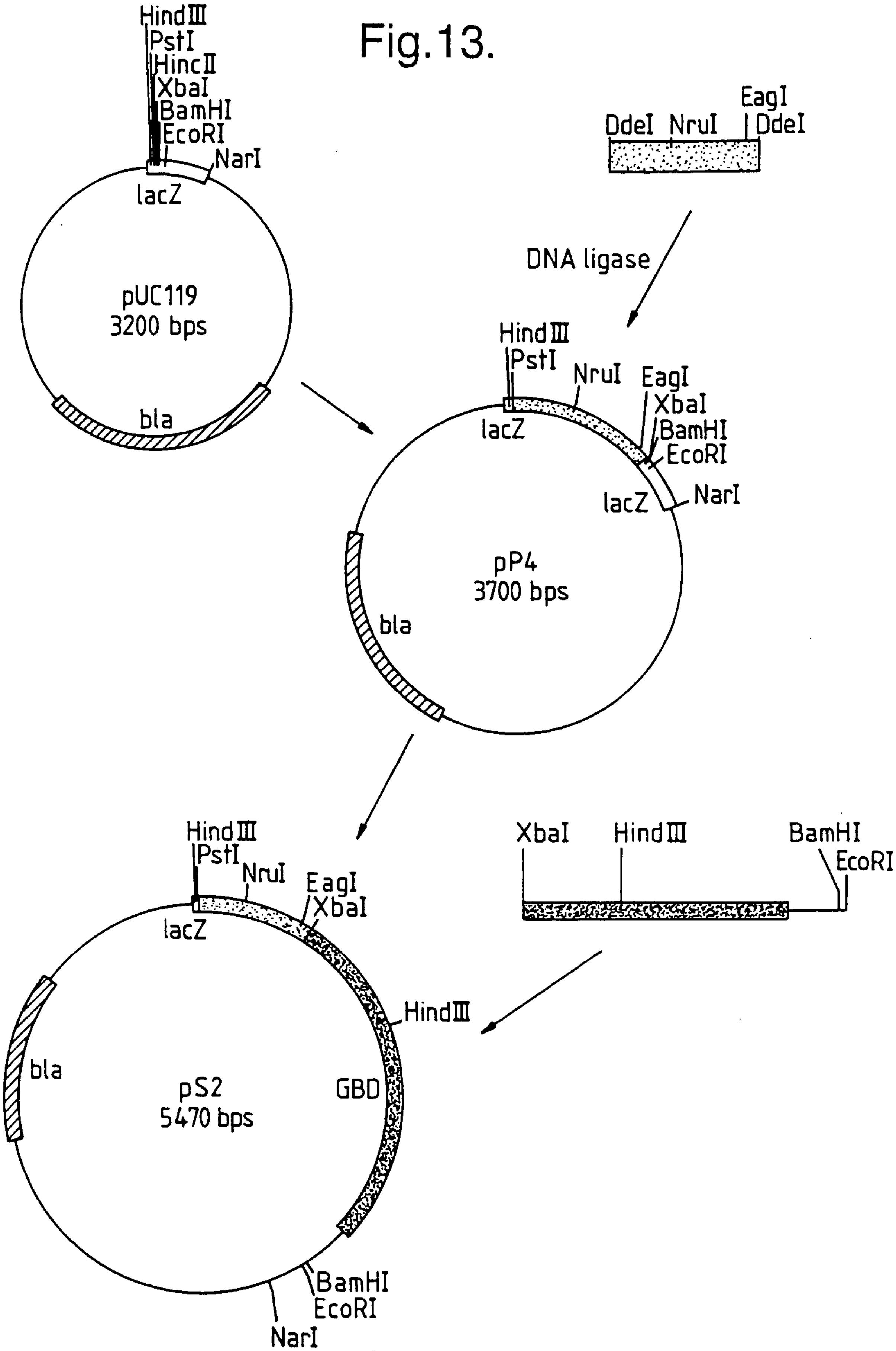

HindIII
PstI
HincII
XbaI
BamHI
EcoRI
NarI
lacZ
pUC119
3200 bps
bla
EagI
DdeI
NruI
DdeI
DNA ligase
HindIII
PstI
NruI
EagI
XbaI
BamHI
EcoRI
lacZ
lacZ
NarI
pP4
3700 bps
bla
HindIII
PstI
NruI
EagI
XbaI
lacZ
XbaI
HindIII
BamHI
EcoRI
HindIII
bla
pS2
5470 bps
GBD
BamHI
EcoRI
NarI

Fig.14.

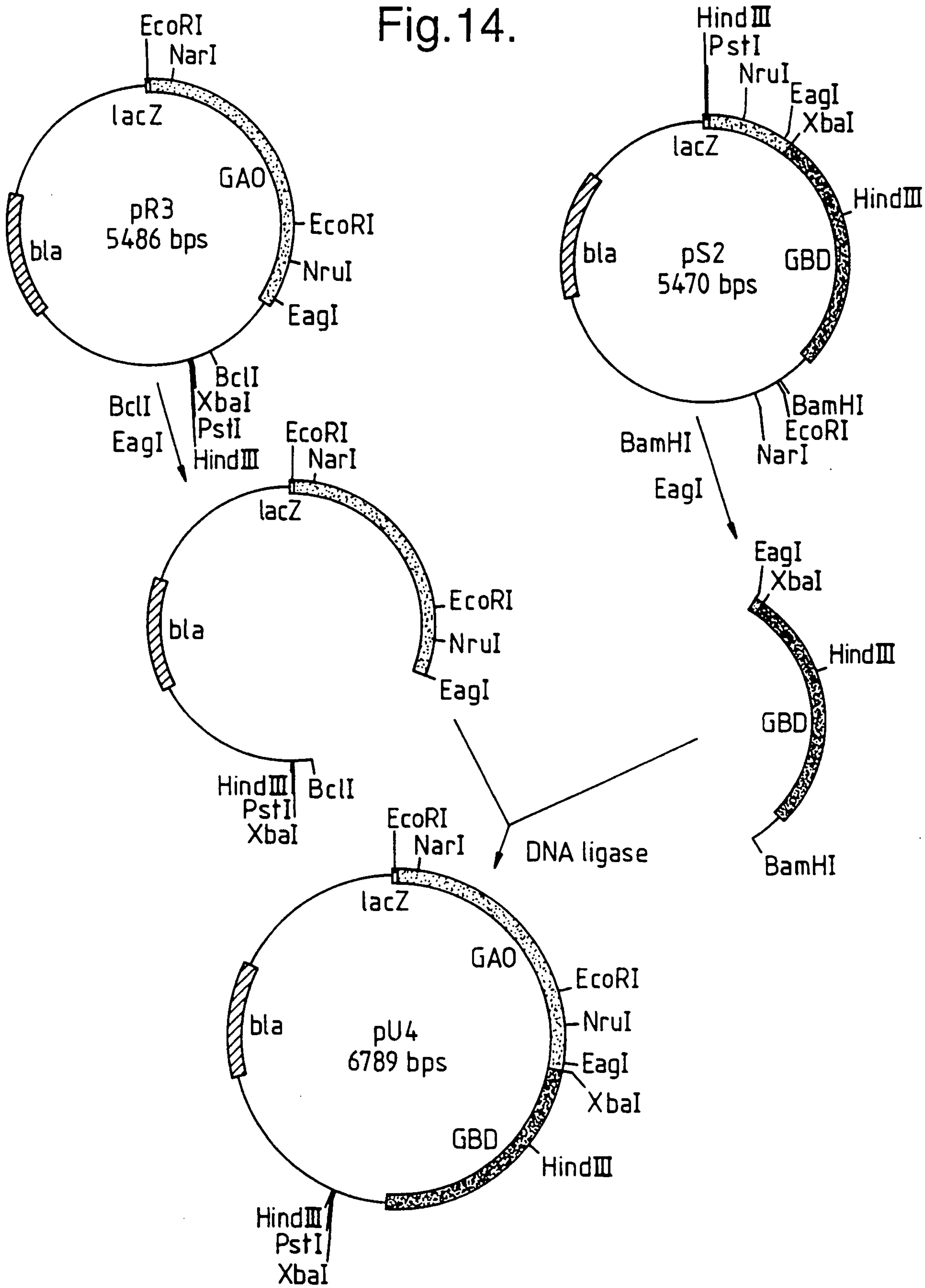

EcoRI
NarI
lacZ
GAO
pR3
5486 bps
bla
EcoRI
NruI
EagI
BclI
XbaI
PstI
HindIII
BclI
EagI
HindIII
PstI
NruI
EagI
XbaI
lacZ
HindIII
bla
pS2
5470 bps
GBD
BamHI
EcoRI
NarI
BamHI
EagI
EcoRI
NarI
lacZ
bla
EcoRI
NruI
EagI
HindIII
PstI
XbaI
BclI
EagI
XbaI
HindIII
GBD
BamHI
DNA ligase
EcoRI
NarI
lacZ
GAO
EcoRI
NruI
EagI
XbaI
bla
pU4
6789 bps
GBD
HindIII
HindIII
PstI
XbaI

Fig.15.

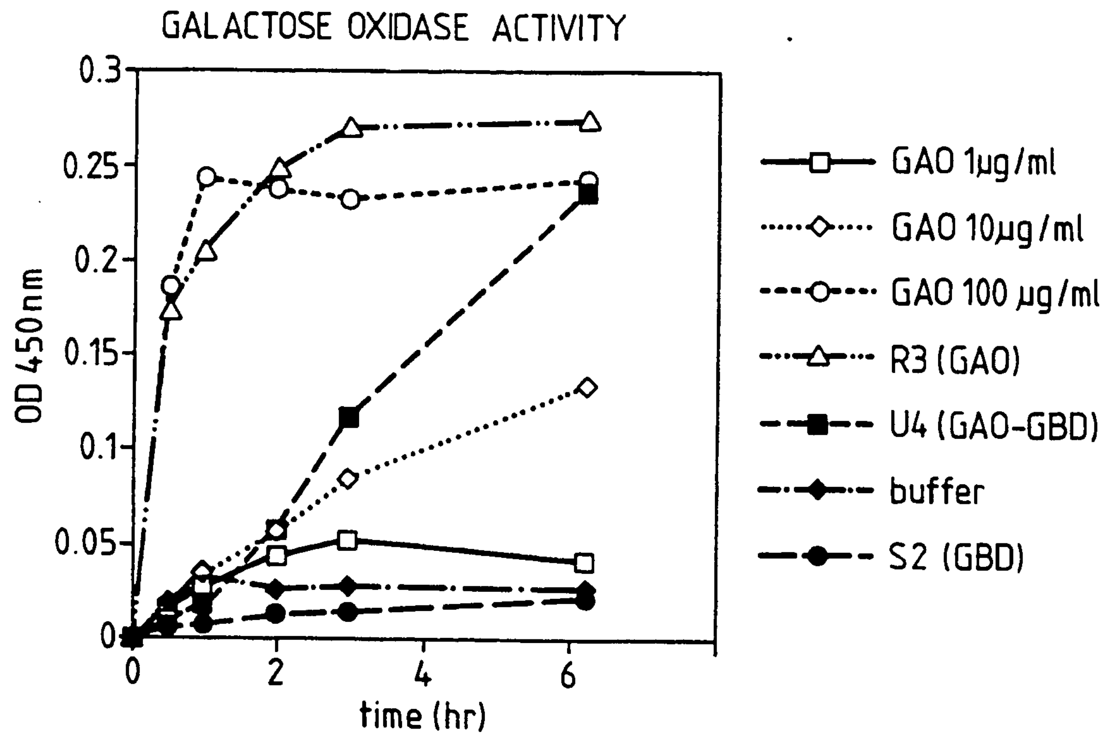
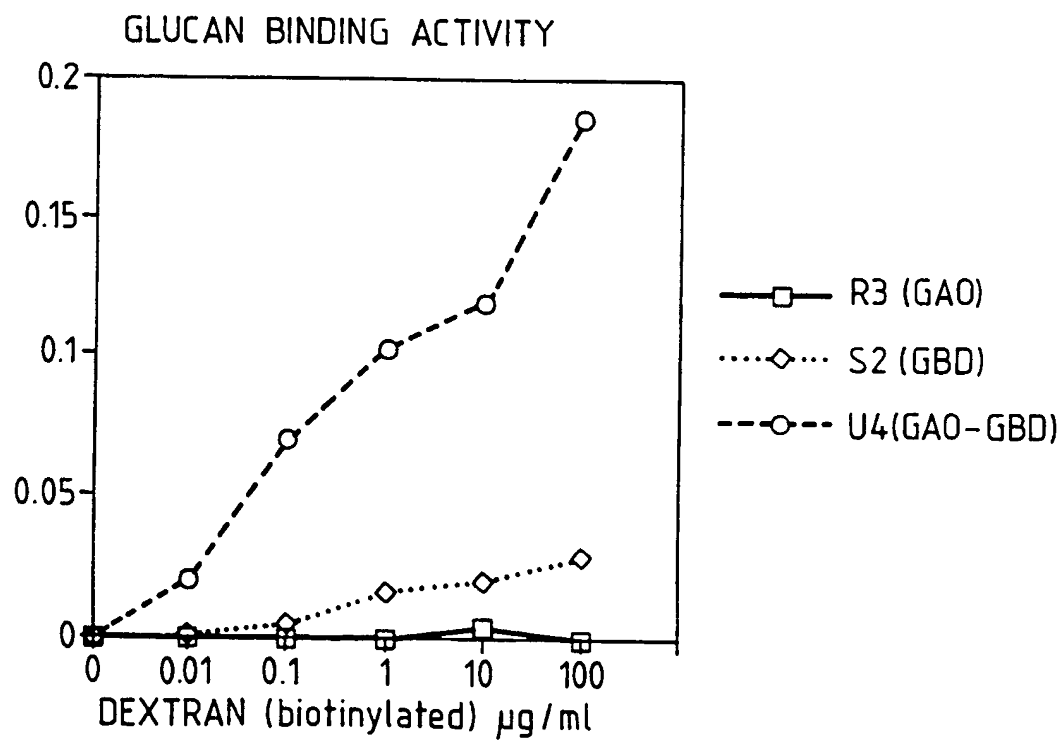

GALACTOSE OXIDASE ACTIVITY
0.3
0.25
0.2
0.15
0.1
0.05
0
OD 450nm
0
2
4
6
time (hr)
GAO 1µg/ml
GAO 10µg/ml
GAO 100 µg/ml
R3 (GAO)
U4 (GAO-GBD)
buffer
S2 (GBD)

Fig.16.


GLUCAN BINDING ACTIVITY
0.2
0.15
0.1
0.05
0
0
0.01
0.1
1
10
100
DEXTRAN (biotinylated) µg/ml
R3 (GAO)
S2 (GBD)
U4(GAO-GBD)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/01070

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N9/10 C07K19/00 A61K7/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 22341 (FORSYTH DENTAL INFIRMARY FOR CHILDREN) 11 November 1993 see page 3, line 2 - line 15 see page 7, line 23 - line 35 see page 8 ---	1,7,11
X	WO,A,93 21331 (UNIVERSITY OF BRITISH COLOMBIA) 28 October 1993 see pages 8,11,16,19 and example 6. ---	1,2,5,7, 8
X	INFECT. IMMUN., vol. 61, no. 11, - November 1993 pages 4689-4695, CHIA J-S. ET AL. 'Inhibition of glucosyltransferase activities of Streptococcus mutans by a monoclonal antibody to a subsequence peptide' see the whole document --- -/--	1,2,7,11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 1995	29.09.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gac, G

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 07979 (CENTER FOR INNOVATIVE TECHNOLOGY) 13 June 1991 see page 4 - page 7 ---	1,7,11
A	EP,A,0 334 467 (KANEBO LTD) 27 September 1989 see the whole document ---	11-14
A	J. BACTERIOL., vol. 174, no. 17, - September 1992 pages 5639-5646, NAKANO ET AL. 'Mechanism of Streptococcus mutans Glucosyltransferases : hybrid-enzyme analysis' see the whole document -----	1-14

1

Information on patent family members

Intern. al Application No
PCT/GB 95/01070

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9322341	11-11-93	CA-A- 2134761	11-11-93
		EP-A- 0638092	15-02-95
		JP-T- 7506374	13-07-95
WO-A-9321331	28-10-93	US-A- 5340731	23-08-94
		EP-A- 0635059	25-01-95
WO-A-9107979	13-06-91	EP-A- 0502099	09-09-92
		JP-T- 5503420	10-06-93
EP-A-334467	27-09-89	JP-A- 1190635	31-07-89
		CA-A- 1325192	14-12-93
		DE-D- 68921908	04-05-95
		DE-T- 68921908	27-07-95
		KR-B- 9410861	18-11-94
		US-A- 5439680	08-08-95
		US-A- 5281524	25-01-94

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB95/01070

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 14
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 14 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.